EQUINE PROTOZOAL MYELOENCEPHALITIS VACCINE

BACKGROUND OF THE INVENTION

Equine protozoal myeloencephalitis (EPM) is a debilitating neurologic disease of equines which can affect the brain, the brain stem, spinal cord or any combination of these three areas of the equine's central nervous system. EPM is caused by the protozoan parasites *Sarcocystis neurona* or *Neospora hughesi*.

A horse of any age, breed or gender may be affected by EPM. The disease has been reported in two-month olds, as well as thirty-year olds. In fact, any horse demonstrating neurologic abnormalities may be infected. Clinical signs of a condition depend upon the location of the organism within the central nervous system. These signs include weakness, malposition of a limb, muscle atrophy, spinal ataxia or the like. A severely EPM-affected horse may go down and be unable to rise. Lameness not traceable to orthopedic disease or any combination of the aforementioned signs may occur in early or less severe infections.

Initially EPM was thought to only be caused by *Sarcocystis neurona*. The opossum (*Didelphis virginiana*) has been identified as the definitive host for this agents. The intermediate host for this organism is still

unknown. The horse ingests feed which has been contaminated with opossum fecal material containing *Sarcocystis neurona* sporocysts. These sporocysts then excyst in the intestinal epithelium of the intermediate and incidental hosts. In the case of the intermediate host, the merezoites would encyst in the tissues of the host forming sarcocysts. In the case of the aberrant host, the *Sarcocystis neurona* multiply in the Central Nervous System (spinal cord) and fail to encyst. In horses, the only observed forms of *Sarcocystis neurona* have been the meront or merozoite.

Recently *Neospora hughesi* has been identified as a second organism which will cause the EPM clinical disease. *Neospora hughesi* will not only infect the spinal cord as *Sarcocystis neurona* does, but will also colonize the brain. At this point in time the definitive and intermediate hosts for *Neospora hughesi* remain unknown. It is believed that fecal contamination of horse feed or water with sporulated oocysts is the route of horse infection. The oocysts will release tachyzoites which will infect cells as do the merezoites of *Sarcocystis neurona*.

In both cases the horse is an aberrant dead-end host and infectious forms of the parasite are not passed from horse to horse or from an infected horse to a definitive or true intermediate host.

There is currently no vaccine or approved animal drug product available for the effective treatment of EPM. The currently available treatments are expensive, of limited efficacy and may include adverse side effects such as anemia, abortion, diarrhea, low white blood cell

counts or the like. There remains an unfulfilled need for treatment for EPM-afflicted equines, particularly horses, which is effective, convenient to administer and useful for the reduction of resistant strains.

Therefore, it is an object of this invention to provide an immunogenically active component useful for the prevention or amelioration of EPM.

It is another object of this invention to provide a vaccine composition suitable for use in equines against infection and disease caused by the protozoan parasites *Sarcocystis neurona* and/or *Neospora hughesi*.

It is a further object of this invention to provide a method for the prevention or amelioration of EPM disease in equines that need such protection. Other objects and features of the invention will become apparent from the detailed description set forth herein below.

SUMMARY OF THE INVENTION

The present invention provides an immunogenically active component which comprises inactivated *Sarcocystis neurona* cells or inactivated *Neospora hughesi* cells; DNA derived therefrom; or a mixture; or in combination with other vaccine components.

The present invention further provides an immunogenically active component which comprises a member selected from the group consisting of merozoite antibody inducing, inactivated *Sarcocystis neurona* cells; tachyzoite antibody inducing, inactivated *Neospora hughesi* cells; a merozoite or tachyzoite antibody inducing antigen derived or extracted from said cells;

DNA derived from said cells capable of inducing a merozoite or tachyzoite antibody immune response; and a mixture thereof.

Further provided is a vaccine composition which comprises an effective immunizing amount of at least one of the above-said immunogenically active components and a pharmacologically acceptable carrier.

Still further provided is a vaccine composition which comprises a) an effective amount of one immunologically active component selected from merozoite antibody inducing, inactivated *Sarcocystis neurona* cells; a merozoite antibody inducing antigen derived or extracted from said cells; DNA derived from said cells capable of inducing a merozoite antibody immune response, and a mixture thereof; b) an effective amount of a second immunologically active component selected from tachyzoite antibody inducing, inactivated *Neospora hughesi* cells; a tachyzoite antibody inducing antigen derived or extracted from said cells; DNA derived from said cells capable of inducing a tachyzoite antibody immune response; and a mixture thereof; and c) a pharmacologically acceptable carrier.

The present invention also provides a method for the prevention or amelioration of infection or disease caused by *Sarcocystis neurona* protozoa in equines that need such protection. The method for the prevention or amelioration of EPM infection or disease in equines comprises administering to said equine an immunogenically active component which comprises a member selected from the group consisting of merozoite antibody inducing, inactivated *Sarcocystis neurona* cells; tachyzoite

antibody inducing, inactivated *Neospora hughesi* cells; a merozoite or tachyzoite antibody inducing antigen derived from said cells; DNA derived from said cells capable of inducing a merozoite or tachyzoite antibody immune response; or a mixture thereof; and, optionally, a pharmacologically acceptable carrier.

Also provided is a method for the cell culture propagation of protozoan parasites, including *Sarcocystis* spp. and *Neospora* spp.

DETAILED DESCRIPTION OF THE INVENTION

Sarcocystis neurona or *Neospora hughesi* protozoa are the causative agents of equine protozoal myeloencephalitis (EPM) disease, which is a serious, and sometimes fatal, neurological disease in equines, particularly horses. EPM symptoms include hypermetria, decreased proprioception, weakness, cranial nerve deficits, general ataxia or the like. The opossum has been identified as the definitive host for these organisms. However an intermediate host is, as yet, unknown. Equines are the aberrant host and apparently become infected when ingesting feed which has been contaminated with the *Sarcocystis neurona* or *Neospora hughesi* protozoans via opossum fecal contamination. EPM disease when untreated will progress from initial numbness of limbs to final central nervous system destruction, resulting in death. Heretofore, there were no known vaccination or immunization treatments available against EPM.

Surprisingly, it has now been found that an immunogenically active component which comprises inactivated *Sarcocystis neurona* cells or antigens, subunit proteins or plasmid DNA; inactivated *Neospora hughesi* cells or antigens, subunit proteins or plasmid DNA; or mixtures thereof may be administered in the form of a vaccine composition to prevent or ameliorate EPM disease in equines, particularly horses. Antigens derived from *Sarcocystis neurona* or *Neospora hughesi* may be obtained using conventional procedures such as outer membrane extraction. Plasmid DNA derived from *Sarcocystis neurona* or *Neospora hughesi* may be obtained via isolation from sources such as the fluids or tissues of equine mammalians diagnosed to have EPM. Such sources include cerebral spinal fluid or sections of spinal cord or brain. Alternatively, the precursor of the infectious stage in horses (sporocyst or cyst) may be obtained from feces or intestinal scrapings of opossums or other wild life present in endemic locales. *Sarcocystis* Spp. or *Neospora* SPP. cells, thus obtained, may be maintained in the infected equine or in suitable tissue culture media, such as RPMI 1640 medium or in cells known in the art such as African green monkey kidney (Vero) cells or equine dermal (E. Derm) cells. The *Sarcocystis* Spp. or *Neospora* Spp. protozoa may then be separated from the tissue culture of cell media using conventional techniques such as centrifugation, filtration, or the like. A useful starting isolate for the vaccines of the invention include, for example, for *Sarcocystis neurona*, the isolate designated SN3; other such isolates are those known as SN1, SN2, SN4, SN5, SN6, UCD-1, UCD-2 and UCD-3

and are variously available from the University of Kentucky, Dr. J.P Dubey at the USDA, U. of California - Davis, Oregon State University, the University of Missouri and others. A culture of one such *Sarcocystis neurona* isolate designated SNg, originally isolated from the intestinal scrapings of the opossum and confirmed to be a representative *Sarcocystis neurona* by PCR, was deposited with the ATCC on January 25, 2001, and given ATCC Accession No. PTA-2972. A useful starting isolate for the vaccines of the invention include, for example, for *Neospora hughesi*, the isolate designated NEQ1; another such isolate is that known as NE1, which has been described by Antoinette Marsh *et al*, Journal of Parasitology, 84 (5), 1998, pp983-991. A culture of one such Neospora hughesi isolate has been deposited with the ATCC and given ATCC Accession No. 209622 (NE1) as disclosed in US 6,071,737. Surprisingly, it has now been found that protozoan parasites such as *Sarcocystis* spp. or *Neospora* Spp. may be propagated in increased yield and increased active viability via cell culture propagation by growing suitable cells to a monolayer having a confluency of about 80%-100% in a growth media; decanting the growth media; refeeding the cells with fresh growth media; inoculating the cells with merozoites or tachyzoites; after 4-12 days, decanting the growth media; and refeeding the inoculated cells a second time with growth media. Cells suitable for use in the method of the invention include cells such as E. Derm cells, Vero cells, Maiden Darby Bovine Kidney (MDBK) cells, Canine Monocyte (DH82) cells, Mouse Monocyte (P388) cells, Fetal Rhesus Monkey Kidney cells, Feline Kidney (FKCU) cells,

Maiden Darby Canine Kidney (MDCK) cells, Baby Hamster Kidney (BHK21) cells, or the like, preferably E. Derm or Vero cells, more preferably E. Derm cells.

In actual practice, the cells are grown to a monolayer having at least 80%, preferably 90%-100%, confluency in a growth media such as MEM with 0.05% lacalbumin hydrosylate (LAH) or Optimem (LTI, Gaithersburg, MD) supplemented with 10% fetal bovine serum, iron fortified fetal calf serum or donor serum. When the cell monolayer has been formed, the culture is decanted to remove the original growth media, the cells are refed with a growth media such as RPMI 1640 with no antibiotics and 25 μM hepes buffer supplemented with 1% sodium pyruvate/2-mercaptoethanol solution having a pH of 6.8 - 7.8, preferably 7.2 - 7.4, and up to 10% fetal bovine serum. The refed cells are then inoculated with merozoites or tachyzoites, held for 4 to 12 days and decanted to remove the growth media. The culture is then refed a second time with growth media as described above and monitored for disease progression. When a level of cytopathology of >60% is obtained, the culture may be harvested.

The thus-obtained whole cell isolates of *Sarcocystis* Spp. or *Neospora* Spp. protozoa may be inactivated by conventional inactivating means, for example chemical inactivation using chemical inactivating agents such as binary ethyleneimine, beta-propiolactone, formalin, merthiolate, gluteraldehyde, sodium dodecyl sulfate, or the like or a mixture thereof, preferably formalin. Said

whole cell isolates may also be inactivated by heat or psoralen in the presence of ultraviolet light.

As used herein the term "immunogenically active" designates the ability to stimulate an immune response, i.e., to stimulate the production of antibodies, particularly humoral antibodies , or to stimulate a cell-mediated response. For example, the ability to stimulate the production of circulating or secretory antibodies or the production of a cell-mediated response in local mucosal regions, i.e., intestinal mucosa, peripheral blood, cerebral spinal fluid or the like.

The immunogenically active component of the invention may be incorporated into liposomes using known technology such as that described in Nature, 1974, 252, 252-254 or Journal of Immunology, 1978, 120, 1109-13. Further, the immunogenically active component of the invention may be conjugated to suitable biological compounds such as polysaccharides, peptides, proteins, or the like, or a combination thereof.

Advantageously, the immunogenically active component of the invention may be formulated as a vaccine composition in dosage unit form to facilitate administration and insure uniformity of dosage. The vaccine composition of the invention comprises an effective immunizing amount of the immunogenically active component described hereinabove, a pharmacologically acceptable carrier and optionally an immunogenically stimulating adjuvant. The effective immunizing amount of the immunogenically active component may vary and may be any amount sufficient to evoke an immune response. Amounts wherein the dosage unit comprises at least about

$1x10^4$ inactivated *Sarcocystis* Spp. cells or *Neospora* Spp. cells or a mixture thereof, preferably at least about $1x10^6$ cells, are suitable.

As used in the specification and claims, the term "immunogenically stimulating adjuvant" designates a compound which is capable of potentiating or stimulating the immune response in a subject animal when administered in combination with the immunogenically active component of the invention. Examples of an immunogenically stimulating adjuvant suitable for use in the vaccine composition of the invention include: surfactants such as hexadecylamine, octadecylamine, lysolecithin, dimethyl dioctadicyl ammonium bromide, N,N-dioctadecyl-N'-N-bis(2-hydroxyethyl-propane diamine), methoxyhexadecylglycerol, PLURONIC polyols, saponin, Quil® A, or the like; polyanions such as pyran, dextran sulfate, polynucleotide complex of polyinosinicpolycytidylic acid, polyacrylic acid, carboxypolymethylenes and carboxyvinyl polymers such as CARBOPOL®, aluminum hydroxide, aluminum phosphate, or the like; peptides such as muramyl dipeptide, dimethyl glycine, tuftsin or the like; oil emulsions; immunomodulators such as interleukin-1, interleukin-2, interleukin-12, GM-CSF or the like; or a combination thereof. A preferred immunogenically stimulating adjuvant suitable for use in the vaccine composition of the invention is a mixture of squalane and a polyoxyethylene-polyoxypropylene block copolymer (e.g., Pluronic® L121, BASF, Parsippany, NJ) capable of forming small liposomes. The immunogenically stimulating adjuvant may be present in the vaccine composition of the

invention in wt/wt amounts of about 1% to 50%, preferably about 5% to 20%.

Pharmacologically acceptable carriers suitable for use in the vaccine composition of the invention may be any conventional liquid carrier suitable for veterinary pharmaceutical compositions, preferably a balanced salt solution suitable for use in tissue culture media.

In addition to the immunogenically active component as active ingredient, it is contemplated the vaccine composition of the invention may also contain other active components such as an antipathogenic component directed against rabies virus, Eastern equine encephalitis virus, Western equine encephalitis virus, Venezuelan equine encephalitis virus, equine herpes virus such as EHV-1 or EHV-4, *Ehrlichia risticii*, *Streptococcus equi*, tetanus toxoid, or the like or a combination thereof.

The inventive vaccine composition may be administered parenterally, for example, intramuscularly, subcutaneously, intraperitoneally, intradermally or the like, preferably intramuscularly; or said composition may be administered orally or intranasally.

The vaccine composition of the invention is useful for the prevention or amelioration of EPM infections in equine that need such protection. In actual practice, the vaccine composition of the invention is administered parenterally, orally, or intranasally, preferable parenterally , more preferably intramuscularly, in effective amounts according to a schedule determined by the time of potential exposure to infective *Sarcocystis* Spp. or *Neospora* Spp. sporocysts. In this way, the

treated animal may have time to build immunity prior to natural exposure. For example, a typical treatment schedule may include parenteral administration, preferably intramuscular injection, at least 5-8 weeks prior to potential exposure. At least two administrations are preferred, for example one at about 8 weeks and a second at about 3 weeks prior to potential exposure of the treated animal.

For a more clear understanding of the invention, the following examples are set forth below. These examples are merely illustrative and are not understood to limit the scope or underlying principles of the invention in any way. Indeed, various modifications of the invention, in addition to those shown and described herein, will become apparent to those skilled in the art from the following examples and the foregoing description. Such modifications are also intended to fall with the scope of the appended claims.

Unless otherwise noted, all parts are parts by weight.

EXAMPLE 1

A - Vaccine preparation

An equine spinal cord isolate of *Sarcocystis neurona* is obtained from a horse which has been diagnosed to have EPM. The isolate is cultivated in multiple cultures of E. Derm cells in RPMI tissue culture medium at 37°C. These merozoite harvests are counted at the time of harvest and then inactivated by means of addition of a 10% formalin solution to a final concentration of 0.05%.

This is allowed to inactivate at 37°C for a period of no less than 48 hours.

To remove unnecessary serum proteins associated with tissue culture the harvests are pooled and diafiltrated/concentrated against 0.01M phosphate buffered saline to a level of 3.14×10^7 merozoites per mL.

The vaccines are formulated by suspending the appropriate volume of inactivated cells in an adjuvant containing 1-20% by volume of a metabolizable oil adjuvant per 1 mL dose, e.g., 5% of the stock adjuvant described herinbelow.

B - Formulation of A Preferred Stock Adjuvant

A preferred adjuvant for use in the present invention was prepared according to the following formulation:

Polyoxyethylene-polyoxypropylene block copolymer (e.g., Pluronic® L121, BASF, Parsippany,NJ)	20 ml
Squalane (e.g, Kodak, Rochester, NY)	40 ml
Polyoxyethylenesorbitan monooleate (e.g., Tween® 80, Sigma Chemical, St. Louis, MO)	3.2 ml
buffered salt solution (e.g., D-V PAS Solution, Ca, Mg free)	936.8 ml

The ingredients are mixed and homogenized until a stable mass or emulsion is formed. Prior to homogenization, the ingredients or mixture can be autoclaved. The emulsion may be further sterilized by filtration. Formalin may be added up to a final concentration of 0.2%. Thimerosal may be added to a final dilution of 1:10,000.

EXAMPLE 2

Antibody response to intramuscular injection of vaccine

In this evaluation, horses that are found to be naive to *Sarcocystis neurona* merozoite antigen by means of Indirect Fluorescent Antibody (IFA) testing are employed. Horses are randomly divided into four groups: one group of nine horses are administered vaccine at the level of $1x10^5$ merozoites per dose; a second group of twenty-one horses are administered vaccine blended at $1x10^6$ merozoites per dose; a third group of ten horses are administered vaccine at $1x10^7$ merozoites per dose; and a fourth of group of ten horses are maintained as non-vaccinated environmental controls. Treated horses are given a first dose of vaccine according to the group to which they are assigned. At twenty-one days following administration of the first dose, a second dose of the same vaccine is administered. All horses are bled for serum at the time of administration of the first and second dose and at weekly intervals through 28 days post second dose administration.

In this evaluation, the vaccine compositions contain formalin-inactivated, E. Derm cell line-grown *Sarcocystis neurona* merozoites with an adjuvant system. The method of serologic measurement of antibodies is conducted by IFA. The IFA is run using Vero cell line-grown *Sarcocystis neurona* merozoites to eliminate anti-E. Derm antibody titers.

The serological data is shown in Table I below, wherein: 0 DPV 1 designates day zero, pre vaccination; 0 DPV 2 designates day zero, post vaccination; 7 DPV 2

designates day 7, post vaccination; and 14 DPV 2 designates day 14, post vaccination.

As can be seen from the data on Table I, treated horses from all groups showed significant increases in antibodies to *Sarcocystis neurona* merozoites while the control horses maintained a low to non-existent antibody level. The level of response in the horses that received vaccine was dependent upon the level of antigen in the vaccine that they received.

Table I

EPM (*Sarcocystis neurona*) Dose Titration IFA Serology

No.	Vaccine	Antigen Load	0 DPV 1	0 DPV 2	7 DPV 2	14 DPV 2
1	1	1x10^5 mer.	<1:10	1:80	1:640	1:800
2	1	1x10^5 mer.	<1:10	1:480	1:480	1:3200
3	1	1x10^5 mer.	<1:10	1:40	1:320	1:800
4	1	1x10^5 mer.	<1:10	1:160	1:320	1:3200
5	1	1x10^5 mer.	<1:10	1:320	1:480	1:3200
6	1	1x10^5 mer.	<1:10	1:320	1:160	1:1600
7	1	1x10^5 mer.	<1:10	1:40	1:80	1:400
9	1	1x10^5 mer.	<1:10	1:320	1:160	1:800
10	1	1x10^5 mer.	<1:10	1:480	1:640	1:4800
GMT			**<1:10**	**1:211**	**1:300**	**1:1550**
11	2	1x10^6 mer.	<1:10	1:640	1:960	1:4800
12	2	1x10^6 mer.	<1:10	1:960	1:1920	1:4800
13	2	1x10^6 mer.	<1:10	1:160	1:240	1:800
14	2	1x10^6 mer.	<1:10	1:640	1:1280	1:4800
15	2	1x10^6 mer.	<1:10	1:1280	1:2560	1:12800
16	2	1x10^6 mer.	<1:10	1:320	1:1280	1:4800
17	2	1x10^6 mer.	<1:10	1:320	1:640	1:3200
18	2	1x10^6 mer.	<1:10	1:1280	1:960	1:3200
19	2	1x10^6 mer.	<1:10	1:160	1:960	1:4800
20	2	1x10^6 mer.	<1:10	1:320	1:640	1:800
21	2	1x10^6 mer.	<1:10	1:320	1:640	1:4800
22	2	1x10^6 mer.	<1:10	1:960	1:1920	1:4800
23	2	1x10^6 mer.	<1:10	1:640	1:1280	1:4800
24	2	1x10^6 mer.	<1:10	1:1280	1:2560	1:3200

Table I, (Continued)

No.	Vaccine	Antigen Load	0 DPV 1	0 DPV 2	7 DPV 2	14 DPV 2
25	2	1x10^6 mer.	<1:10	1:640	1:1280	1:4800
26	2	1x10^6 mer.	<1:10	1:1280	1:960	1:3200
27	2	1x10^6 mer.	<1:10	1:1280	1:1280	1:3200
28	2	1x10^6 mer.	<1:10	1:1280	1:1280	1:4800
29	2	1x10^6 mer.	<1:10	1:2560	1:960	1:4800
30	2	1x10^6 mer.	<1:10	1:2560	1:2560	1:3200
31	2	1x10^6 mer.	<1:10	1:1280	1:1280	1:3200
GMT			**<1:10**	**1:734**	**1:1147**	**1:3704**
32	3	1x10^7 mer.	<1:10	1:1280	1:1920	1:3200
33	3	1x10^7 mer.	<1:10	1:640	1:1280	1:4800
34	3	1x10^7 mer.	<1:10	1:640	1:1280	1:1600
35	3	1x10^7 mer.	<1:10	1:2560	1:2560	1:4800
36	3	1x10^7 mer.	<1:10	1:2560	1:5120	1:4800
37	3	1x10^7 mer.	<1:10	1:2560	1:5120	1:4800
38	3	1x10^7 mer.	<1:10	1:1280	1:1280	1:12800
39	3	1x10^7 mer.	<1:10	1:2560	1:7680	1:19200
40	3	1x10^7 mer.	<1:10	1:1920	1:2560	1:19200
41	3	1x10^7 mer.	<1:10	1:1280	NS	1:12800
GMT			**<1:10**	**1:1429**	**1:2296**	**1:6630**
42	4	Control	<1:10	<1:10	<1:10	<1:10
43	4	Control	<1:10	<1:10	<1:10	<1:10
44	4	Control	<1:10	<1:10	<1:10	<1:10
45	4	Control	<1:10	<1:10	<1:10	<1:10
46	4	Control	<1:10	<1:10	<1:10	<1:10
47	4	Control	<1:10	<1:10	<1:10	<1:10
48	4	Control	<1:10	<1:10	<1:10	<1:10
49	4	Control	<1:10	<1:10	<1:10	<1:10

Table I, (Continued)

No.	Vaccine	Antigen Load	0 DPV 1	0 DPV 2	7 DPV 2	14 DPV 2
50	4	Control	<1:10	<1:10	<1:10	<1:10
51	4	Control	<1:10	<1:10	<1:10	<1:10
GMT			**<1:10**	**<1:10**	**<1:10**	**<1:10**

Sample	Type	Material	IFA Titer
MSU1	Positive	Control Sera	1:800
MSU2	Positive	Control Sera	1:800
Blakely	Positive	Control Sera	1:400
Sport	Positive	Control Sera	1:160

EXAMPLE 3

Plaque reduction effect as determined by serum of vaccinated horses

In this evaluation, an assay is performed to determine if the *Sarcocystis neurona* antibody found by IFA in the serum of EPM vaccinated horses would have a neutralizing effect on *Sarcocystis neurona* merozoites at varying levels of the organism.

Horse serum samples are collected at 14 days post second vaccination from the Example 2 study group 3, which received vaccine containing $1x10^7$ merozoites per dose; and the samples are pooled. Duplicate sets of this serum are diluted 1:2 to a 1.0 mL volume and are mixed with 1.0 mL volumes of varying levels of viable *Sarcocystis neurona* merozoites, resulting in a final serum dilution of 1:4. The organism (merozoite) levels used 1:10 are $2.5x10^5$, 1:100 are $2.5x10^4$, and 1:1000 are

$2.5x10^3$ merozoites per mL. Duplicate sets of serum/organism tubes are set up using a serum pool from the group 4 non-vaccinated horses to stand as a negative control group for comparison. The 2.0mL organism/serum mixtures are incubated for 1 hour at 37°C and then added to 25 cm^2 of E. Derm cells with the appropriate media to support *Sarcocystis neurona*. At 14 days post inoculation all flasks are fixed using a 10% formalin/crystal violet stain and are counted for the number of plaques present in each flask.

There was a marked reduction in the number of plaques observed in the flasks which had received the serum from the group 3 vaccinate horses which had been incubated with organism at all organism dilutions when compared to similar flasks which had the non-vaccinated control serum. This data is shown in Table II below.

As can be seen from the data in Table II, the degree of plaque reduction in every case of the vaccinated horse serum pools exceeded 70%.

TABLE II

Sarcocystis neurona Plaque Reduction Serology

Organism Dilution	Sample	Serum Dilution	Plaques Observed	Average No. of Plaques	Percent Reduction[2]
1:10	Vaccine 3	1:4	87	97.0	89.22
1:10	Vaccine 3	1:4	107		
1:100	Vaccine 3	1:4	16	14.0	73.33
1:100	Vaccine 3	1:4	12		
1:1000	Vaccine 3	1:4	2	1.5	85.00
1:1000	Vaccine 3	1:4	1		
1:10	Controls	1:4	TNTC[1]	TNTC	NA
1:10	Controls	1:4	TNTC		
1:100	Controls	1:4	55	52.5	NA
1:100	Controls	1:4	50		
1:1000	Controls	1:4	12	10.0	NA
1:1000	Controls	1:4	8		

[1]TNTC estimated to be 900-1000 plaques (900 used for calculations).

[2]Percent Reduction as compared to the number of plaques in the corresponding control serum dilution plaque count.

EXAMPLE 4

Vaccine preparation

Neospora hughesi is obtained from the brain or spinal column of a horse that has been diagnosed to have

EPM. The isolate is cultivated in multiple cultures of E. Derm or Vero cells in RPMI tissue culture medium at 37°C. The tachyzoites harvested are counted at the time of harvest and then inactivated by means of addition of a 10% formalin solution to a final concentration of 0.05%. This is allowed to inactivate at 37°C for a period of no less than 48 hours.

To possibly remove unnecessary serum proteins associated with tissue culture the harvests are pooled and may be diafiltrated/concentrated against 0.01M phosphate buffered saline to a suitable level of tachyzoites per mL for final vaccine formulation.

The vaccine is formulated with antigen as in Example 1.

EXAMPLE 5

Cell culture propagation of *Sarcocystis* spp. and *Neospora* spp.

Equine dermal (E. derm) cells that have been grown to achieve a monolayer of 90-100% confluency are decanted to remove the original cell growth media (OptiMEM supplemented with 10% fetal bovine serum). The E. derm cells are then refed with RPMI 1640 media supplemented with 1% sodium pyruvate/2-mercaptoethanol[1] solution having a pH of 7.2 - 7.4 and 10% fetal bovine serum and

[1]The sodium pyruvate/2-mercaptoethanol solution consists of 0.175 mL 2-mercaptoethanol and 0.600g sodium pyruvate in 500 mL of RPMI 1640 media (pH 7.2 - 7.4) which has been sterile filtered.

inoculated with viable merozoites or tachyzoites. After 4-12 days, the resultant culture is decanted to remove the growth media and then refed a second time with RPMI 1640 media supplemented with 1% sodium pyruvate/2-mercaptoethanol solution[1] having a pH of 7.2 - 7.4 and 2% - 10% bovine fetal serum. The resultant culture is then monitored for disease progression and when a level of greater than 60% cytopathology is obtained the culture is harvested.